ARYL HYDROCARBON HYDROXYLASE INDUCTION IN HUMAN LYMPHOCYTE CULTURES BY 2,3,7,8-TETRACHLORODIBENZO-p-DIOXIN

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ABSTRACT. Aryl hydrocarbon (benzo(a)pyrene) hydroxylase activity is induced in cultured human lymphocytes by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) at a concentration in the growth medium 40 to 60 times less than the concentration of 3-methylcholanthrene (MC) necessary for maximal hydroxylase induction. In cultured lymphocytes from 19 individuals, the extent of hydroxylase induction by TCDD or MC ranged between 1.7- and 2.9-fold. Those individuals having (presumably under genetic control) lower basal and MC-inducible hydroxylase activities in their lymphocytes also have lower TCDD-inducible hydroxylase activity. Although preliminary in nature, the data concerning the observed variance of expression of hydroxylase induction more closely fit a unimodal, polygenic (i.e. 2 or more genes) pattern rather than a trimodal (single gene) form of inheritance.

INTRODUCTION. The possible importance of aromatic hydroxylations of polycyclic hydrocarbons, drugs, and other environmental agents mediated by the membrane-bound monooxygenases to chemical carcinogenesis, pharmacology, and toxicology has been recently reviewed (1). Genetic differences in the induction of one such monooxygenase activity, the aryl hydrocarbon hydroxylase system, have been demonstrated in fetal mouse cell cultures (2), in mice (3-5), and in cultured human lymphocytes (6). An increased incidence of 3-methylcholanthrene-initiated sarcomas in mice (7-10) and, more recently, bronchiogenic carcinoma in man (11) has been highly correlated with the genetic "responsiveness" of the individual (i.e. the mouse or human having the hydroxylase activity most inducible by aromatic hydrocarbons).

Recent studies have shown (12) that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a toxic contaminant formed during the commercial

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synthesis of the herbicide 2,4,5-trichlorophenoxyacetic acid, is approximately 30,000 times more potent than 3-methylcholanthrene (MC) as an inducer of aryl hydrocarbon hydroxylase activity in rat liver. Moreover, the hydroxylase activity in liver, kidney, bowel, lung, and skin of so-called "nonresponsive" mice is induced fully by TCDD, but not by MC or β-naphthoflavone (13,14). Because TCDD is metabolized so slowly in the rat, the biological half-life of this potent inducer is about 17 days (15) and the induced hydroxylase activity and associated cytochrome P_1450 remain elevated for more than 35 days (12). Thus, TCDD may become a serious environmental contaminant for man; evidence for the appearance of this toxic agent in the food chain has already been reported (16). Obvious questions arise. What dose of TCDD will be hazardous to man? What are the consequences of prolonged TCDD-induced aryl hydrocarbon hydroxylase activity in various human tissues? Do those individuals having genetically lower basal and MC-inducible hydroxylase activities also have lower TCDD-inducible hydroxylase activity in their lymphocytes? This last question is shown to be the case in this report.

EXPERIMENTAL PROCEDURE. Venous blood (usually 40cc) was collected in heparinized syringes from apparently healthy volunteers. No volunteer was currently on any medications. The whole blood was centrifuged at 200 x g for 15 min and 9 ml of the uppermost plasma-rich fraction was layered onto a 6ml ficoll-hypaque gradient (specific gravity 1.080) (17). At least 60% of the total lymphocyte yield - with the least number of contaminating red blood cells - exists in this plasma-rich fraction. In order to procure the remaining 40% of the lymphocytes, the remaining whole blood was diluted 20% with whole medium (RPMI #1640, 0.20 M HEPES buffer, 20% fetal calf serum and 50μg of gentamicin per ml (all products from Microbiological Associates, Inc., Bethesda, Maryland)) and similarly applied in 9ml aliquots to 6ml ficoll-hypaque gradients. Following a 1,000 x g centrifugation for 45 min, the lymphocyte "bands" were collected and combined, the lymphocytes were washed twice in whole medium, counted, and diluted to a concentration of about 0.8×10^6 cells per ml of whole medium. Five-ml cultures were made and bacto-phytohemagglutinin M (Difco Laboratories, Detroit, Michigan) and pokeweed mitogen (Grand Island Biological Co., Grand Island, New York) were added to final concentrations of 1% each, in order to "activate" the lymphocytes (increased metabolism, lymphoblast formation, and/or cell division usually occurs between one and three days in culture).

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Stock solutions of 240µg of TCDD (kindly provided by Dr. A. Poland, University of Rochester School of Medicine and Dentistry, Rochester, New York) per ml of p-dioxane and 8.0mg of MC (Sigma Chemicals of St. Louis, Misouri) per ml of dimethylsulfoxide were diluted appropriately. The MC was purified by recrystallization from benzine before use. Dimethylsulfoxide and p-dioxane - at concentrations of 0.5% and 0.1%, respectively, or less - were not cytotoxic and did not affect the hydroxylase induction; the basal hydroxylase activity in this study was routinely determined in cultured lymphocytes exposed to 0.1% p-dioxane. Following incubation (37° with 5% CO_2) for 72 hours, the cells were treated with TCDD, MC, or p-dioxane alone in a volume of 0.01 ml. Twenty-four hours later, the cultures were agitated to break up clumps of cells, and the cells were counted in a Fisher autocytometer II cell counter (Fisher Instruments, Pittsburgh, Pennsylvania). The cells were then pelleted by centrifugation at 1,000 x g for 10 min and resuspended in 0.10 M Tris-chloride buffer, pH 7.8.

The enzyme assay and the Lowry protein determination were performed on the whole cells by means of published procedures (2, 3, 6). One unit of aryl hydrocarbon hydroxylase activity is defined (2-4) as that amount of enzyme catalyzing per min at 37° the formation of hydroxylated product causing fluorescence equivalent to that of 1 pMole of 3-hydroxybenzo(a)pyrene. Both duplicate and quadruplicate determinations were performed at different times, and the variability was almost always 10% or less. In this report, aryl hydrocarbon hydroxylase specific activity is expressed in either units per mg of cellular protein or units per 10^6 lymphocytes.

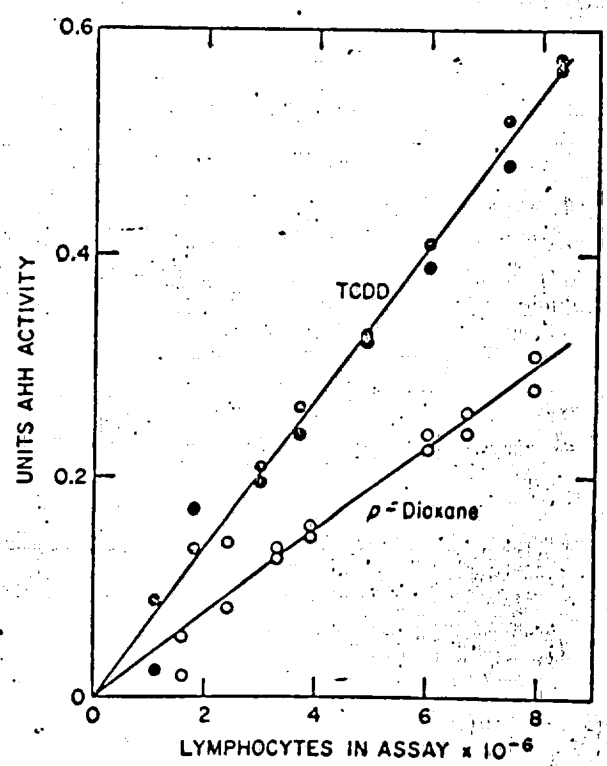
The data is given in terms of the inducible ratio (IR) which is the ratio of hydroxylase activity in TCDD- or MCA-treated lymphocytes to the enzyme activity in cultures treated with the solvent alone. The use of this parameter cancels out much of the normally occuring day-to-day variations associated with mitogen-activation, for regardless of the degree of activation, only the relative increase associated with TCDD or MCA treatment is being measured.

RESULTS AND DISCUSSION. Two major difficulties with the assay were encountered initially: a) large variations in the number of "mitogen-activated" lymphocytes at the end of 4 days in culture, and b) a high nonspecific fluorescence in the zero-time samples. The first problem was alleviated by increasing the purity of the lymphocyte preparations - by means of the ficoll-hypaque gradients as outlined above- thereby resulting in greater than 85% small lymphocytes. Since the enzyme activity appears to be associated with "mitogen-activated" lymphocytes, the highest yield of lymphocytes relative to other cell types should result in the most re-

producible data. Also, since the data are expressed in terms of enzyme activity per given number of cells, the more reproducibly the number of lymphocytes can be quantitated, the more reproducible the data should be. The second problem was largely corrected by the use of more cells per assay tube, use of longer incubation periods (we have found the assay to be linear for at least 60 min) use of glassware cleaned carefully with soap containing no brighteners, and use of highly purified reagents. Although we found (Fig. 1) that the assay was linear at lymphocyte concentrations between 1×10^6 and 8×10^6 cells per assay tube, reproducibility was difficult with 3×10^6 cells or less. In fact, when 2×10^6 cells or less are assayed in the usual 1.0ml reaction mixture, we observe in the emission spectrum at about 522nm only a slight shoulder rather than a definitive peak. Currently we routinely use about 4×10^6 lymphocytes per assay tube and incubate

with

FIG. 1



Aryl hydrocarbon hydroxylase (AHH activity as a function of number of lymphcytes used in the enzyme assay. The closed and open circles represent cells treated with 100nM TCDD and p-dioxane (0.1%), respectively, in the culture medium.

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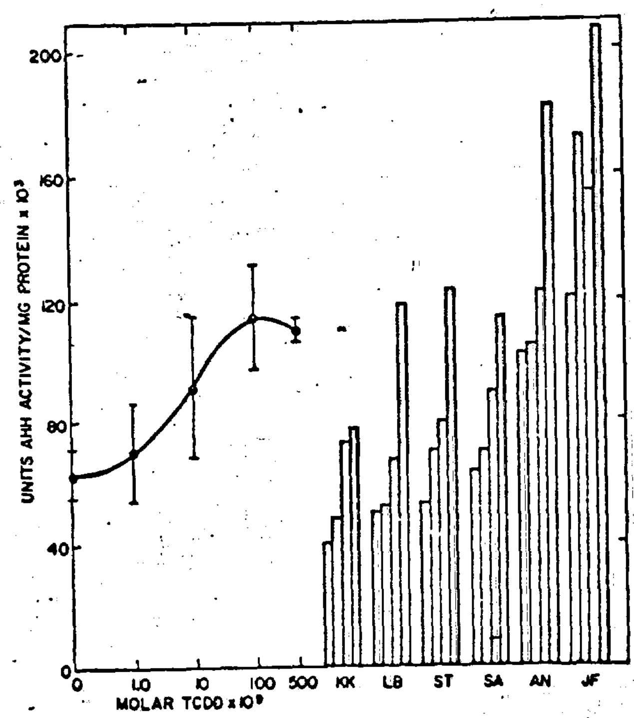
the substrate benzo[a]pyrene for 45 min. With the use of Kimble disposable 125 x 15 mm glass tubes (Kimble Glass Division, Owens-Illinois Glass Co., Toledo, Ohio) and spectral grade acetone and hexane (Fisher Scientific, Silver Spring, Maryland), fluorescence in the zero-time sample is now less than 10% of the fluorescence representing the basal enzyme activity. The zero-time sample contains the complete reaction mixture plus cells and benzo[a]pyrene, but to which cold acetone-hexane has been added prior to incubation. Similar values are obtained if the benzo[a]pyrene plus reaction mixture are incubated for the prescribed length of time and the cells are then added after the addition of cold acetone-hexane.

Fig. 2 shows the hydroxylase induction in response to varying concentra-

FIG. 2



Aryl hydrocarbon hydroxylase (AHH) induction by TCDD. At left is a dose-response curve representing 5 separate experiments on lymphocytes taken at different times from the same individual. The circles and brackets denote the mean + S.E.M. Despite the large variations from one experiment to the next, the maximal extent of enzyme induction by TCDD in each experiment was reasonably constant (i.e. 1.4 to 1.7-fold) and approximated that by 1.5 μM MC.

At right are histograms representing six different individuals, whose initials appear below. The four bars, from left to right, depict the hydroxylase activity in lymphocytes treated with 0, 1.0, 10, and 100 nM TCDD, respectively. 100 nM represents about 33 ng per ml. Maximal induction in the six individuals, from left to right, is about 2.0, 2.4, 2.3, 1.8, 1.8, and 1.7, respectively.

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tions of TCDD in the growth medium. Fifty per cent of the maximal induction (ED_{50}) was achieved with about 8 nM TCDD. The histograms in Fig. 2 and the data in Table I illustrate that, in lymphocytes from any given individual, the higher the basal hydroxylase activity, the higher the TCDD-inducible hydroxylase activity. Whether the hydroxylase specific activity is expressed in units per mg of cellular protein (Fig. 2) or in units per 10^6 cells (Table I), our conclusion is basically the same.

TABLE I

Effect of TCDD on aryl hydrocarbon hydroxylase induction in cultured human lymphocytes from four individuals

Dose of TCDD (nM)	Dose of TCDD (ng/ml)	R.K. SA[a]	R.K. IR[b]	D.A. SA	D.A. IR	G.G. SA	G.G. IR	M.W. SA	M.W. IR
0	0	0.039	(1.0)	0.042	(1.0)	0.050	(1.0)	0.050	(1.0)
0.3	0.10	0.043	1.1	0.046	1.1	0.055	1.1	0.050	1.0
3.0	1.0	0.055	1.4	0.067	1.6	0.100	2.0	0.065	1.3
30	10	0.077	2.0	0.088	2.1	0.130	2.6	0.140	2.8
300	100	0.079	2.0	0.094	2.2	0.141	2.8	0.145	2.9

[a]Specific activity (SA) is expressed as units of hydroxylase activity per 10^6 cells. These values represent the mean specific activity of 2 to 5 separate experiments performed at different times on lymphocytes from the same individuals.

[b]Inducibility ratio (IR) is the ratio of hydroxylase activity in TCDD-treated lymphocytes to the enzyme activity in cultures treated with the solvent p-dioxane alone.

Table II shows that the index of inducibility is about the same in lymphocytes from any given individual, when maximal inducing doses of either TCDD or MC are present in the culture medium. We estimate that the optimal inducing doses of TCDD and MC are about 30 nM and 1.5 μM, respectively; thus, TCDD is about 40 to 60 times more potent than MC as an inducer of hydroxylase activity in cultured human lymphocytes. This is in marked contrast to the 30,000-fold

TABLE II

Comparison of TCDD and MC as inducers of aryl hydrocarbon hydroxylase activity in cultured human lymphocytes from thirteen individuals

Volunteer's initials	Basal hydroxylase activity	TCDD (10 ng/ml or 30 nM)		MC (0.4 µg/ml or 1.5 µM)	
		SA[a]	IR[b]	SA	IR
P.G.	0.031	0.056	1.8	0.064	2.1
T.R.	0.033	0.059	1.8	0.064	1.9
K.T.	0.039	0.067	1.7	0.067	1.7
R.K.	0.039	0.078	2.0	0.070	1.8
A.L.	0.039	0.078	2.0	0.084	2.2
D.A.	0.042	0.088	2.1	0.084	2.0
S.G.	0.045	0.090	2.0	0.106	2.4
G.M.	0.048	0.098	2.0	0.092	1.9
H.R.	0.048	0.123	2.6	0.140	2.9
G.G.	0.050	0.132	2.6	0.126	2.5
M.W.	0.050	0.137	2.7	0.140	2.8
A.V.	0.053	0.140	2.6	0.137	2.6
C.M.	0.056	0.140	2.5	0.154	2.8

[a]Specific activity (SA) of the basal and induced enzyme is expressed as units of hydroxylase activity per 10^6 cells. These values represent the mean specific activity of at least two experiments performed at different times on lymphocytes from each individual.

[b]Inducibility ratio (IR) is the ratio of hydroxylase activity in TCDD- or MC-treated lymphocytes to the enzyme activity in cultures treated with p-dioxane alone. The correlation coefficients r for the relationship between the basal and induced hydroxylase activities from these 13 individuals are 0.82 and 0.73 for TCDD and MC, respectively ($P<0.01$ for both). This significant correlation between the basal and MC-induced enzyme activities is in agreement with the data of Kellermann et al. (6). However, our basal and inducible levels of hydroxylase activity in cultured lymphocytes are about 2 to 4 times lower than those reported by Kellermann and coworkers (6). We have since found that different levels of basal and inducible hydroxylase activities occur when lymphocytes from the same blood sample are grown in different lots of fetal calf serum, bacto-phytohemagglutinin M, and poke weed mitogen. The studies in this report were all performed with the same lots of these materials. For any large-scale comparison of genetic expression, therefore, the same lots of fetal calf serum, bacto-phytohemagglutinin M, and pokeweed mitogen should be constantly used throughout the entire study.

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difference in potency between TCDD and MC in rat liver (12). This difference between 50-fold in culture and 30,000-fold in the intact animal is not understood and is under further investigation. It is possible that this effect reflects different binding affinities and/or tissue distribution differences between these two inducers in the intact animal that are not operant in cell culture. Hence, TCDD may be far more potent in man than what we observe in cultured human lymphocytes.

Several points concerning the assay of hydroxylase activity in lymphocytes should be emphasized. (i) The variance in the specific enzyme activity from leukocytes of the same individual from one week to the next is quite significant (e.g. the brackets in the dose-response curve of Fig. 2 represent the standard error of the mean). (ii) With either MC or TCDD as the inducer, we do not find "distinct classes of 'low,' 'intermediate,' and 'high' inducibility"--as was described by Kellermann and coworkers (6) with MC as the inducer. In fact, the greatest extent of hydroxylase induction we have yet found among 32 individuals (unpublished data) has been a factor of 2.9-fold. From the apparent Hardy-Weinberg distribution reported in the Houston population (6), we would have expected to see 2 or 3 individuals in the "high inducibility" group but we have found none in this "class." (iii) The enzyme induction even among various inbred strains of mice appears to involve at least 2, and probably more than 2, nonlinked genetic loci (5)—rather than the one locus as was first postulated (3, 4). These observations would lead us to believe that the observed variance of expression of hydroxylase induction in an outbred population--such as man--more closely fits a unimodal, polygenic, rather than a trimodal (single-gene), pattern of inheritance. This hypothesis is presently under further investigation.

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We have shown in this report a positive correlation between basal enzyme activity and the enzyme levels maximally inducible by either TCDD or MC. This threshold difference in response to aromatic hydrocarbon inducers has also been repeatedly observed with the various inbred strains of mice (2, 3, 13, 14). It is therefore possible that the more highly "responsive" individuals in the human population exposed to TCDD are more susceptible to any effects produced by prolonged elevated levels of induced hydroxylase activity. TCDD itself is not a potent carcinogen in mice; however, the synergistic action of TCDD with MC produces cancer in different strains of mice in direct proportion to the degree of elevation of the induced hydroxylase activity and associated cytochrome P_1450 content (R.E. Kouri, A.P. Poland, and D.W. Nebert, manuscript in preparation). The facts that TCDD induces aryl hydrocarbon hydroxylase activity in man and that this toxic compound is present in relatively high levels in certain parts of the world (16) suggest that, in addition to the short-term risk of TCDD because of toxic (18-24) and teratogenic (25, 26) properties, there may be considerable long-term risk because of possible synergism in chemically initiated tumorigenesis.

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